THE JOURNAL OF INVESTIGATIVE DERMATOLOGY
Vol. 48, No. 4

Printed in U.S.A.

TYROSINASE INHIBITOR IN FORTNER'S AMELANOTIC AND MELANOTIC MALIGNANT MELANOMA*

GERALDINE J. ZDYBEK SATOH, PH.D. AND YUTAKA MISHIMA, M.D., PH.D.

Although information has accumulated regarding the nature of tyrosinase and its role in melanin pigmentation, very little is known as to why there is almost complete absence of melanization in the melanosome of amelanotic malignant melanoma whereas the reverse is true of melanotic malignant melanoma.

In 1946 Rothman and co-workers postulated that the natural regulation of melanin formation may depend upon organic sulfhydryl compound inhibition of tyrosinase, and demonstrated an inhibitory factor in human epidermis which could be released by specific sulfhydryl poisons (1). Similar studies were reported by Flesch in 1949 (2). Riley (3) and Hirsch (4) reported an inhibitor of dopa oxidase activity in mouse melanoma. Identification and characterization of such inhibitors has not been well defined.

The following is a report of a preliminary study of tyrosinase inhibitor found in amelanotic and melanotic malignant melanoma of the Syrian golden hamster.

METHODS AND MATERIALS

Fortner's amelanotic malignant melanoma type 3 and Fortner's melanotic malignant melanoma type 1 (5) were obtained from Joseph G. Fortner, M.D., Sloan Kettering Institute, New York, and transplanted thereafter in our laboratory. Amelanotic melanoma type 3 had been preserved by Fortner at low temperature under a nitrogen atmosphere prior to our transplantation.

Syrian golden hamsters used for transplantation were obtained from Lakeview Hamster Colony, Newfield, New Jersey, the same strain as that used by Fortner.

Non-necrotic melanomas were homogenized in 0.25M sucrose-0.02M phosphate buffer using a Teflon head hand homogenizer, centrifuged at 700 × g to remove cell debri and then centrifuged at 12,000 × g to obtain a soluble fraction. Temperatures were maintained at 2°C. during this preparation. All tumors were so processed unless otherwise specified.

Studies of tyrosinase enzyme kinetics were carried out in 1 cm cells in a Zeiss PMQ II spectrophotometer by the oxidation of L-3,4 dihydroxyphenylalanine (dopa) followed by measuring the initial rate of increase in optical density at 475 millimicrons. The final reaction mixture contained 0.5 μ mole/ml dopa and 35 μ mole/ml phosphate buffer, pH 6.8.

RESULTS

Addition of soluble fraction of amelanotic melanomas to that of the soluble fraction from melanotic melanoma caused marked inhibition of melanotic melanoma tyrosinase activity as measured by the spectrophotometric dopachrome formation utilizing L-dopa as substrate. This is illustrated in Fig. 1. Line A represents the soluble fraction of melanotic melanoma equivalent to 0.06 g, where 5 μ mole of dopa is converted per hour per gram melanoma. Line B represents the equivalent amount of melanotic melanoma soluble fraction (0.06 g) to which the soluble fraction of amelanotic melanoma fraction is added equivalent to 0.06 g amelanotic melanoma, where 3.36 μ mole dopa was converted per hour per gram tumor or 33% decrease in activity. The initial lag period becomes 15 minutes whereas there was a 10 minute lag with melanotic melanoma alone. Adding amelanotic fraction equivalent to 0.12 g of amelanotic melanoma reduced the activity to 1.12 μ mole per hour per gram melanotic melanoma or 78% decrease in activity, as represented in line C. The lag period then became 25 minutes.

Investigations were pursued to elucidate whether this inhibitor was amelanotic melanoma specific. Inhibitory activity of soluble fractions equivalent to 0.04 g, 0.08 g, and 0.12 g of original melanomas were measured utiliz-

* From the Departments of Dermatology, Wayne State University, Detroit General Hospital, Detroit, Michigan and Veterans Administration Hospital, Dearborn, Michigan.

This investigation was supported by Public Health Service Research Grant No. CA-05580-05 from the National Cancer Institute, No. AM-07981-03 from the National Institute of Arthritis and Metabolic Diseases, and in part by the Michigan Cancer Foundation and the Receiving Hospital Research Corporation.

Received for publication May 4, 1966.

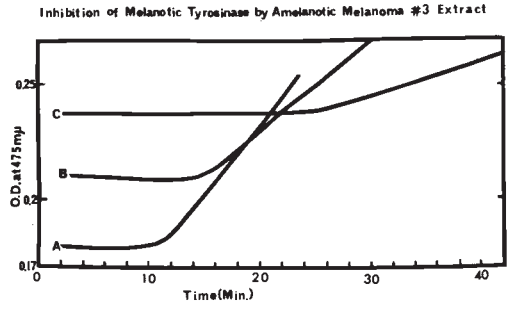

FIG. 1: Inhibition of melanotic tumor tyrosinase by amelanotic melanoma fraction plotted as optical density at 475 mμ against minutes. Line A represents soluble fraction of melanotic melanoma (0.06 g); B represents soluble fraction of melanotic tumor (0.06 g) + amelanotic soluble fraction equivalent to 0.06 g melanoma; C represents soluble fraction of melanotic melanoma (0.06 g) + amelanotic soluble fraction equivalent to 0.12 g melanoma.

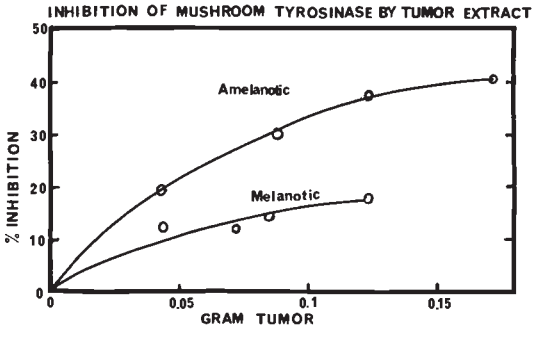

FIG. 2: The percent of inhibition of mushroom tyrosinase by melanotic and amelanotic soluble fraction plotted against gram wet weight of melanoma.

ing L-dopa as substrate and crystallized mushroom tyrosinase as enzyme source. The linear portion of mushroom tyrosinase from 30 seconds to 1 minute and 30 seconds was used, during which time interval no enzyme activity was contributed by melanotic fraction since the lag phase of melanotic fraction was at least 5 minutes. Fig. 2 indicates that the percent of inhibition of mushroom tyrosinase by amelanotic melanoma fraction is considerably greater than that of melanotic melanoma, there being 18% inhibition by amelanotic fraction as compared to 11% by melanotic for 0.04 g melanoma respectively, 29% by amelanotic compared to 13% by melanotic for 0.08 g melanoma respectively, and 37% by amelanotic as compared with 17% by melanotic for 0.12 g melanoma. Therefore, although inhibitor effect is demonstrated by melanotic melanoma, the inhibition is significantly more pronounced in amelanotic melanoma.

In order to study further the properties of this inhibitor, melanomas were homogenized in 0.02 M phosphate buffer and a 12,000 x g soluble fraction prepared. These fractions were dialyzed against distilled water at pH 7.0 overnight. The diffusates were flash-evaporated and then tested for their inhibitory effect on mushroom tyrosinase. Fig. 3 shows that both melanotic and amelanotic diffusate equiv-

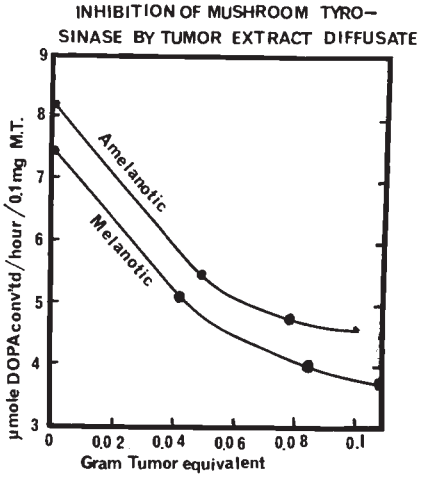

FIG. 3: Inhibition of mushroom tyrosinase by amelanotic and melanotic tumor diffusates plotted as μ mole dopa converted/hour/0.1 milligram mushroom tyrosinase against gram wet weight tumor equivalent.

INHIBITION OF MELANOTIC HOMOGENATE TYROSINASE ACTIVITY

	μmole/hr./g melanotic tumor
.06g melanotic	2.5
.06g melanotic + .13g amelanotic	1.4
.06g melanotic + .2g amelanotic dialysate	2.5
.06g melanotic + .2g equiv. amelanotic diffusate	1.1
.06g melanotic + .06g equiv. melanotic diffusate	1.0

FIG. 4: Inhibition of melanotic soluble fraction tyrosinase activity by amelanotic soluble fraction, amelanotic and melanotic diffusate, and amelanotic dialysate expressed as μ mole dopa converted/hour/gram melanotic tumor equivalent.

alent to 0.04 g, 0.08 g, and 0.1 g original melanoma had quite similar inhibitory effect; therefore, 0.04 g had 33% inhibition, 0.08 g had 43% inhibition, and 0.1 g had 45% inhibition. The dialysate had no inhibitory effect toward mushroom tyrosinase or melanoma soluble fractions. Furthermore, the inhibitor from melanotic melanoma appears to be bound as its inhibitory effect is much less than that exhibited by amelanotic melanoma whereas after dialysis the inhibitor from both melanomas exerts very similar inhibitory effect.

Fig. 4 illustrates inhibition of soluble fraction tyrosinase from melanotic melanoma by soluble fraction from amelanotic melanoma, and melanotic and amelanotic inhibitor isolated from the diffusate after dialyzing 12,000 x g melanoma homogenates. The dialysate of amelanotic fraction no longer exhibits an inhibitory effect. Of most interest is the fact that once inhibitor of melanotic melanoma is freed by dialysis it is a potent inhibitor of melanotic homogenate.

In summary, the following properties are attributed to inhibitor obtained by dialysis of 12,000 x g homogenates of both melanotic and amelanotic melanoma:

1. Heat stable.
2. Dialyzable.
3. Not extractable with acid butanol, ether, chloroform, benzene.
4. Inhibitory effect on mushroom tyrosinase unchanged by addition of Cu^{+2}, therefore this substance appears not to be a sulfhydryl compound.
5. Kinetic studies reveal the inhibitors to be competitive.

Using mushroom tyrosinase as an enzyme source, the Michaelis-Menten constant of mushroom tyrosinase was found to be 3.3×10^{-4}M, the K_i of melanotic inhibitor 5.5×10^{-4}M, and the K_i of amelanotic inhibitor 4.2×10^{-4}M. The K_m value of mushroom tyrosinase was close to that found for tyrosinase from melanotic melanoma.

Further studies are now in progress to isolate and identify these inhibitors.

CONCLUSIONS

The defects in melanogenesis in amelanotic melanoma may be attributed in part to disturbed tyrosinase synthesis and the presence of inhibitors since melanosome formation (6) and active tyrosine release utilizable as substrate has been shown in amelanotic melanoma (7).

Fortner's amelanotic melanoma type 3 exhibited no tyrosinase activity in 20 cases presently studied. Furthermore, removing inhibitor from amelanotic fractions by dialysis failed to reveal tyrosinase activity.

The characteristic properties of inhibitor found in both melanotic and amelanotic melanoma are heat stability, dialyzable, not a sulfhydryl compound, and competitive inhibition.

REFERENCES

1. Rothman, S., Drysa, H. F. and Smiljanic, A. M.: Inhibitory action of human epidermis on melanin formation. Proc. Soc. Exp. Biol. Med., *62:* 208, 1946.
2. Flesch, P.: Inhibitory action of extracts of mammalian skin on pigment formation. Proc. Soc. Exp. Biol. Med., *70:* 136, 1949.
3. Riley, V., Hobby, G. and Burk, D.: Pigment Cell Growth, pp. 231–266, (M. Gordon, ed.), New York, Academic Press, 1953.
4. Hirsch, H. M.: Pigment Cell Biology, pp. 327–358, (M. Gordon, ed.), New York, Academic Press, 1959.
5. Fortner, J. G., Mohy, A. G. and Schrodt, G. R.: Transplantable tumors of the Syrian golden hamster. Part I: Tumors of the alimentary tract, endocrine glands, and melanomas. Cancer Res., *21:* 161, 1961.
6. Mishima, Y.: Subcellular activities in the ontogeny of nevocytic and melanocytic melanomas. Read at the 16th Annual Symposium in the Biology of Skin; the Pigmentary System. Portland, Oregon. April 22–24, 1966. In: Advances in Biology of Skin, Vol. 8. W. Montagna, ed. New York, Pergamon Press, 1967. in press.
7. Satoh, G. J. Zdybek and Mishima, Y.: Subcellular enzyme-substrate relationships between amelanotic and melanotic malignant melanoma. Fed. Proc. Abstract, *25:* 294, 1966.